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(54) Title: LIPOSOMAL CYCLOSPORIN FORMULATIONS AS AGENTS FOR IMMUNOSUPPRESSION AND MULTIPLE DRUG RESISTANT INDICATIONS

(57) Abstract

Improved liposomal encapsulated cyclosporin formulations are disclosed. The liposomes are efficacious as immunosuppressant agents and in the treatment of drug resistant cancers. The formulations include liposomes comprised of a phosphatidylcholine, cholesterol, a phosphatidylglycerol and a cyclosporin. In one embodiment, the mole ratios of phosphatidylcholine, cholesterol, phosphatidylglycerol and cyclosporin are about 21:0.5:3:1 to 21:1.5:3:1 and 24:0.5:3:1 to 24:1.5:31 wherein the liposomes comprise unilamellar vesicles having a size less than 100 nm. In a preferred embodiment, the compositions are stable upon injection into the blood stream of a mammal, preferably a human. In this embodiment, the preferred ratios of PC:chol:PG:CSA are from about 28:1:3:1 to 40:1:3:1. The preferred formulas are PC:chol:DMPG:CSA wherein the PC is HSPC and the molar ratios are: 28:1:3:1, 30:1:3:1, 32:1:3:1, 34:1:3:1, 35:1:3:1, 36:1:3:1, and 40:1:3:1. Also provided is a liposome encapsulated cyclosporin which provides for a cyclosporin which associates to a significant degree with a liposomal/plasma fraction (vs. cell fraction) of blood as a function of time. Liposomes having these properties are comprised of phosphatidylcholine, cholesterol, dimyristoylphosphatidylglycerol and cyclosporin. These liposomes are unilamellar and have a size less than 75 nanometers and are stable in whole mammal blood. Further provided are liposomes having increased therapeutic indices. The liposomes are stable on storage, contain a therapeutically effective amount of a cyclosporin, provide a liposomal cyclosporin formulation having reduced toxicity, and, in the preferred embodiment, provides a liposomal formulation which is stable in whole blood.

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LIPOSOMAL CYCLOSPORIN FORMULATIONS AS AGENTS FOR IMMUNOSUPPRESSION AND MULTIPLE DRUG RESISTANT INDICATIONS

Field of the Invention

This invention relates to the fields of biochemistry and medicine, and in particular to a novel liposomal formulation and process. More specifically, it relates to a liposomal formulation containing the immunosuppressive agent cyclosporine and to its process of manufacture. This invention also relates to a liposomal cyclosporine formulation having reduced toxicity, increased shelf life stability and increased stability in the blood of mammals.

Background of the Invention

The cyclosporins were discovered in 1970 by researchers in attempts to identify new antimicrobial agents. Cyclosporine (also known as cyclosporin A), a potent immuno-suppressive agent, was isolated from two strains of imperfect fungi, *Cylindrocapon lucidum* Booth and *Tolypocladium inflatum* Gams.

Cyclosporins are hydrophobic, neutral, cyclical peptides which have essentially similar chemical and physical characteristics. Cyclosporine is a representative example, and consists of eleven amino acids with a total molecular weight of 1201. Cyclosporine is soluble in methanol, chloroform and ether and essentially insoluble in water. It is supplied for therapeutic purposes as either an intravenous preparation dissolved in a proprietary castor oil and alcohol, or an oral formulation dissolved in Labrophil and olive oil.

Cyclosporine is primarily used for treating allograft patients and has been used in experimental trials for autoimmune diseases. The use of this drug has greatly increased the survival rate of transplant patients since its advent in 1978.

Although cyclosporine is a very useful immunosuppressive agent, it can also be highly toxic when used for prolonged periods of time and/or at high doses, both of which are necessary to ensure graft acceptance. The most severe side effect associated with cyclosporine therapy is drug-induced nephrotoxicity. Vascular interstitial toxicity is the most common form of cyclosporine nephrotoxicity and can manifest itself as three different morphological lesions, occurring either alone or in combination. Although not all of these morphological changes associated with cyclosporine nephrotoxicity are unique to cyclosporine toxicity, if they are observed in combination with one another and there is also a corresponding high level of serum cyclosporine, the damage is probably a result of

cyclosporine toxicity. Some individuals may show some of these adverse reactions at therapeutic doses (5 to 10 mg/kg/day) which produce trough levels of 200-500 ng/ml in whole blood and 20-60 ng/ml in serum. Renal toxicities can be monitored serologically by following the increase in creatinine levels. The increase in creatinine level is probably a direct result of arteriole constriction and blockage which would result in lower glomerular filtration rate and thus an increase in serum creatinine.

There are other adverse side reactions associated with cyclosporine treatment. These occur with varying frequencies depending on the type of transplant. They include symptoms, such as cardiovascular hypertension and cramps, skin hirsutism, gum hyperplasia, diarrhea, nausea, vomiting, hepatotoxicity, hematopoietic alterations including leukopenia and lymphoma, respiratory distress and sinusitis.

Other side effects associated with the intravenous delivery of cyclosporine are due to the intravenous carrier vehicle, Cremophor -El (CreL). CreL is a polyoxyethylated castor oil that is one of the best ionic surfactants used to dissolve lipophilic drugs. The most common of the adverse reactions associated with CreL administration has been anaphylaxis which results from a rapid release of histamine and causes increasing hypertension. It is also believed that part of the nephrotoxicity associated with cyclosporine treatment may be enhanced by CreL deposition and crystal formation within the kidney tubules. Other studies have also shown a decrease in both renal blood flow and creatinine clearance in animals treated with CreL. Riconic acid, a component of CreL, has been shown to cause vasoconstriction which could also be linked to hypertension and decreased glomerular blood flow.

Efforts have been made to eliminate the toxicity of cyclosporine by incorporating the drug into liposomes for purposes of administration, thus eliminating the toxic castor oil vehicle. Liposomes are microscopic delivery vesicles made, in part, from phospholipids which form closed, fluid filled spheres when mixed with water. Phospholipid molecules are polar, having a hydrophilic ionizable head, and a hydrophobic tail consisting of long fatty acid chains. Thus, when sufficient phospholipid molecules are present with water, the tails spontaneously associate to exclude water while the hydrophilic phosphate heads interact with water. The result is a bilayer membrane in which the fatty acid tails converge in the newly formed membrane's interior and the polar heads point in opposite directions toward an aqueous medium. The polar heads at one surface of the membrane point toward the aqueous interior of the liposome. At the opposite surface, the polar heads interact with the surrounding aqueous medium. As the liposomes form, water soluble molecules will be incorporated into the aqueous interior, and lipophilic molecules will tend to be incorporated into the lipid bilayer. Liposomes may be either multilamellar, like an onion with liquid separating many lipid bilayers, or unilamellar, with a single bilayer surrounding an entirely liquid center.

There are many types of liposome preparation techniques which may be employed and which produce various types of liposomes. These can be selected depending on the use, the chemical intended to be entrapped, and the type of lipids used to form the bilayer membrane.

Those parameters which must be considered in producing an optimal liposome preparation are similar to those of other controlled release mechanisms. They are as follows: (1) high percent of chemical entrapment; (2) increased chemical stability; (3) low chemical toxicity; (4) rapid method of production; and (5) reproducible size distribution.

The first method described to encapsulate chemicals in liposomes involved production of multilamellar vesicles (MLVs). The MLV process involves dissolving the lipid components in a suitable solvent, evaporation of the solvent to form a dry lipid film, and hydration of the lipid film with an aqueous medium. The multilamellar vesicles which form are structures having generally more than three concentric bilayers. Lipophilic drugs are incorporated into the MLVs by codissolution of the drugs in the solvent phase, while hydrophilic drugs are entrapped between the bilayers with the hydration buffer. By increasing the length of time of hydration and gentle shaking of the resuspending lipid film, one can achieve a higher proportion of the aqueous phase per mole of lipid, and thus enhance hydrophilic drug encapsulation. The increased entrapment of aqueous buffer can also be achieved by using charged lipids.

Liposomes can also be formed as unilamellar vesicles (UVs), which have diameters up to 2 μm, but generally less than 1 μm.

There are several techniques which are used to produce unilamellar liposomes. Large unilamellar vesicles can be formed using the reverse-phase evaporation method. This is done by removing the organic phase of a sonicated emulsion of phospholipid, buffer and excess organic solvent under pressure. This technique is especially useful for encapsulating large volumes of aqueous phase containing hydrophilic molecules, such as ferritin, 25S RNA or SV-40 DNA. Maximum encapsulation of the LUV aqueous phase (65%) can be obtained if the ionic strength of the aqueous buffer is low (0.01 M NaCl); encapsulation decreases to 20% as the ionic strength is increased to 0.5 M NaCl. The size of the LUVs varies with the lipid and cholesterol content. Vesicles formed from cholesterol and phospholipid with a 1:1 mole ratio, form a heterogeneous size distribution of vesicles with a mean diameter, based upon entrapped volume, of 0.47 μm and a size range of 0.17-0.8 μm. Vesicles prepared from similar phospholipid mixtures lacking cholesterol have a mean size of 0.18 μm and a size range of 0.1-0.26 μm.

The solvent infusion evaporation method can produce both larger or smaller UVs, depending on variations in the technique. To form larger UVs, phospholipids are dissolved in diethylether and injected into a buffer maintained at 55-65°C containing the material to be entrapped or injected. The mixture is kept under vacuum at 30°C. When the

solvent has evaporated, vesicles are formed. The range in diameter of these vesicles is from 0.25-1 μm. This procedure is well suited for entrapment of large molecules.

Smaller unilamellar vesicles can also be formed using a variety of techniques. By dissolving phospholipids in ethanol and injecting them into a buffer, the lipids will spontaneously rearrange into unilamellar vesicles. This provides a simple method to produce UVs which have internal volumes similar to that of those produced by sonication (0.2-0.5 L/mol/lipid). Sonication or extrusion (through filters) of MLVs also results in dispersions of UVs having diameters of up to 0.2 μm, which appear as clear or translucent suspensions.

Another common method for producing small UVs is the detergent removal technique. Phospholipids are solubilized in either ionic or non-ionic detergents such as cholates, Triton X, or n-alkylglucosides. The drug is then mixed with the solubilized lipid-detergent micelles. Detergent is then removed by one of several techniques: dialysis, gel filtration, affinity chromatography, centrifugation or ultrafiltration. The size distribution and entrapment efficiencies of the UVs produced this way will vary depending on the details of the technique used. Also, when protein is entrapped, there is no certainty that once the detergent has been removed, the protein will renature into its native bioactive conformation.

The therapeutic use of liposomes can include the delivery of drugs which are normally very toxic in the free form. In the liposomal form the toxic drug may be directed away from the sensitive tissue and targeted to selected areas. Liposomes can also be used therapeutically to release drugs slowly, over a prolonged period of time, reducing the frequency of administration. In addition, liposomes can provide a method for forming an aqueous dispersion of hydrophobic drugs for intravenous delivery.

When liposomes are used to target encapsulated drugs to selected host tissues, and away from sensitive tissues, several techniques can be employed. These procedures involve manipulating the size of the liposomes, their net surface charge as well as the route of administration. More specific manipulations have included labeling the liposomes with receptors or antibodies for particular sites in the body.

The route of delivery of liposomes can also affect their distribution in the body. Passive delivery of liposomes involves the use of various routes of administration e.g., intravenous, subcutaneous and topical. Each route produces differences in localization of the liposomes. Two common methods used to actively direct the liposomes to selected target areas are binding either antibodies or specific receptor ligands to the surface of the liposomes. Antibodies are known to have a high specificity for their corresponding antigen and have been shown to be capable of being bound to the surface of liposomes, thus increasing the target specificity of the liposome encapsulated drug.

Since the chemical composition of many drugs precludes their intravenous administration, liposomes can be very useful in adapting these drugs for intravenous

delivery. Many hydrophobic drugs, including cyclosporine, fall into this category because they cannot be easily dissolved in a water-based medium and must be dissolved in alcohols or surfactants which have been shown to cause toxic reactions *in vivo*. Liposomes, predominantly composed of lipids, with or without cholesterol, are nontoxic. Furthermore, since liposomes are made up of amphipathic molecules, they can entrap hydrophilic drugs in their interior space and hydrophobic molecules in their lipid bilayer.

In a drive to develop a formula that is both safe and effective, such as required by such agencies as the U.S. Food and Drug Administration, it is desirable to provide formulations that have long shelf life stability. Unilamellar liposomes in many cases tend to aggregate and become larger over time. This is one parameter that indicates that the liposomes are not stable. Of course, other parameters such as drug loss over time (leakage) indicate unstable liposomes.

Thus, for a variety of reasons, having to do primarily with the inability of those of ordinary skill to entrap sufficient cyclosporins in a stable liposomal carrier, a therapeutically effective cyclosporin intercalated liposome product has not been commercially available. It has thus been a desideratum to develop a liposomal cyclosporin containing a formulation which enables a high proportion of the active agent to be incorporated therein, and which is sufficiently stable on the shelf and in the blood of mammals. This invention provides such a product.

It is also desirable to provide for a formulation that offers an optimum therapeutic index, that is, the right combination of high effectiveness and low toxicity. This high therapeutic value can be obtained when the formulation has the proper pharmacological profile based on attainable pharmacokinetics. Such liposomes are those that are found not only to be safe and efficacious, but are those that are stable in the blood of mammals.

Thus, an object of the present invention is to provide an improved liposome encapsulated cyclosporin formulation that has superior shelf life stability and reduced toxicity. It is also an object of the present invention to provide for a liposomal cyclosporin formula which is resistant to the loss of drug in the presence of whole (mammal) blood.

Summary of the Invention

Improved liposomal encapsulated cyclosporin formulations are provided. The liposomes are efficacious as immunosuppressive agents and in the treatment of drug resistant cancers. The formulations include liposomes comprised of a phosphatidylcholine (PC), cholesterol, a phosphatidylglycerol (PG) and a cyclosporin. In one embodiment, the mole ratios of the phosphatidylcholine, cholesterol, the phosphatidylglycerol and the

cyclosporin are about 21:0.5:3:1 to 21:1.5:3:1 and 24:0.5:3:1 to 24:1.5:31 wherein the liposomes comprise unilamellar vesicles having a size less than 100 nm.

Also provided is a liposome encapsulated cyclosporin which provides for a cyclosporin which associates to a significant degree with a liposomal/plasma fraction (vs. cell fraction) of blood as a function of time. Liposomes having these properties are comprised of a phosphatidylcholine, cholesterol, dimyristoylphosphatidylglycerol (DMPG) and a cyclosporin. These liposomes are unilamellar and have a size less than 75 nanometers and are stable in whole mammal blood. Further provided are liposomes having increased therapeutic indices.

In an alternate embodiment, the compositions are stable upon injection into the blood stream of a mammal, preferably a human. In this embodiment, the preferred ratios of PC:chol:PG:cyclosporine A (CSA) are from 28:1:3:1 to 40:1:3:1. The preferred formulas are PC:chol:DMPG:CSAwherein the PC is hydrogenated soy phosphatidylcholine (HSPC) and the molar ratios are: 28:1:3:1, 30:1:3:1, 32:1:3:1, 34:1:3:1, 35:1:3:1, 36:1:3:1, and 40:1:3:1.

The liposomes of the invention as practiced herein are generally prepared by:

(a) dissolving (i) a phosphatidylcholine, (ii) a cholesterol, (iii) a phosphatidylglycerol, and (iv) a cyclosporin in an organic solvent to form a solution wherein the molar ratios of (i):(ii):(iii):(iv) are as described above,

(b) drying the organic solution thus formed to form a solid phase, e.g. a film or powder,

(c) hydrating the solid phase with an aqueous solution having a pH from about 4.5 to about 9.5 to form stable liposomal cyclosporin therapeutic formulations having a mean particle size as described above.

The invention provides a cyclosporin intercalated liposomal formulation which is stable on storage, contains a therapeutically effective amount of active ingredient, provides a liposomal cyclosporin formulation having reduced toxicity, and, in the preferred embodiment, provides a liposomal formulation which is stable in whole blood. Whole blood stability is defined as having $\geq$ 70% of cyclosporin in a plasma/liposomal fraction at four hours and where < 0.5 mg/ml of hemoglobin is released from red blood cells (RBC) to the plasma/liposome fraction at four hours as determined using a modified version of Sigma's Drabkin assay kit. This test shows an improvement of lower concentration hemoglobin released in whole mammal blood of 10-fold to over 200-fold improvement over cyclosporine in cremophor EL.

The hypothesis is that any cyclosporin which is loosely associated with the liposomes would be available to interact with cyclophilin receptors in red blood cell membranes causing RBC lysis and higher concentration of hemoglobin release and higher

cyclosporin in the plasma fraction. Although not being bound to any particular theory, it is hypothesized that the result of this interaction could be manifested as membrane leakiness causing loss of hemoglobin from the red cells to the plasma fraction at quantifiable levels.

Detailed Description of the Invention

As used herein, the term liposome refers to unilamellar vesicles or multilamellar vesicles such as are described in U.S. Patents 4,753,788 and 4,935,171, the contents of which are incorporated herein by reference. The term encapsulation, as used herein, refers to the incorporation of the cyclosporin into the liposome membrane.

Generally, the process of preparing the formulation embodied in the present invention is initiated with the preparation of a solution from which the liposomes are formed. This is done by weighing out appropriate quantities of a phosphatidylcholine, cholesterol, a phosphatidylglycerol and a cyclosporin, preferably cyclosporin A, and dissolving them into an organic solvent, preferably chloroform and methanol in a 1:1 mixture. The solution is evaporated to form a solid lipid phase such as a film or powder, for example, with a rotary evaporator, spray dryer or other means. The film or powder is then hydrated with an aqueous solution having a pH ranging from about 4.5 to about 9.5 to form a liposome dispersion. The preferred aqueous solution for purposes of hydration is a buffered solution such as 9% sucrose/10 mM succinate or 10 mM phosphate buffer. The preferred buffer is 9% sucrose, 10 mM succinate wherein the pH is about 6.5. The lipid film or powder dispersed in buffer is heated from about 25°C to about 65°C, preferably at about 55°C.

Multilamellar liposomes are formed by agitation of the dispersion, preferably through the use of a thin-film evaporator apparatus such as is described in U.S. Patent 4,935,171 or through shaking or vortex mixing. Unilamellar vesicles are formed by the application of a shearing force to an aqueous dispersion of the lipid solid phase, e.g., by sonication or the use of a homogenizing apparatus such as a Gaulin homogenizer or a French press. Shearing force can also be applied using either injection, freezing and thawing, dialyzing away a detergent solution from lipids, or other known methods used to prepare liposomes. The size of the liposomes can be controlled using a variety of known techniques including the duration of shearing force. Preferably, the modified Gaulin homogenizing apparatus described in U.S. Patent 4,753,788 is employed to form unilamellar vesicles having diameters of less than 200 nanometers at a pressure of 3,000 to 10,000 psi and a temperature of about the aggregate transition temperature of the lipids. Methods for the agitation or shearing of lipids to form multilamellar or unilamellar vesicles are known in the art and are not part of this invention *per se*.

Distearoylphosphatidylcholine (DSPC), egg phosphatidylcholine (egg PC), hydrogenated egg phoshatidylcholine (HEPC) and hydrogenated soy phosphatidylcholine (HSPC) are the preferred phosphatidylcholines for use in the invention. The most preferred phosphatidylcholine is HSPC for liposomes in which stability in blood is desired. Other suitable phosphatidylcholines include those obtained from soy beans or other plant sources, or those that are partially or wholly synthetic, such as dipalmitoylphosphatidylcholine. All of these are commercially available.

DMPG is the preferred phosphatidylglycerol for use in the invention. Other negatively charged lipids can be used in combination with or in place of the phosphatidyl glycerol. Other suitable negatively charged lipids for use in the present invention include, but are not limited to, dilaurylphosphatidylglycerol (DLPG), dipalmitoylphosphatidylglycerol (DPPG), and dimyristoylphosphatidic acid (DMPA). All of these are also commercially available.

In one embodiment, the ratios of PC:chol:PG:CSA are from about 21:0.5:3:1 to about 21:1.5:3:1 and from about 24:0.5:3:1 to about 24:1.5:3:1. In this embodiment, the preferred formulation is about 24:1:3:1. The preferred lipids in this embodiment are DSPC and DMPG. The preferred size is below 45 μm and the preferred percent entrapped cyclosporin is about 85% or greater. The liposomes of this embodiment show improved shelf life stability, i.e., no significant visual aggregation for at least 9 weeks at 4°C.

In an alternate embodiment, in which stability in blood is desired, the ratios of PC:chol:PG:CSA are from 28:1:3:1 to 40:1:3:1. The preferred formulas in this embodiment are PC:chol:DMPG:cyclosporin wherein the PC is HSPC and the molar ratios are: 28:1:3:1, 30:1:3:1, 32:1:3:1, 34:1:3:1, 35:1:3:1, 36:1:3:1, and 40:1:3:1.

The preferred cyclosporin for use in the invention is cyclosporine A (CSA). The preferred size for liposomes in which stability in blood is desired is below 50 nm. The preferred percent entrapped cyclosporin is about 85 % or greater. The liposomes of the invention as practiced herein, show improved shelf life stability, i.e., no significant (visual) aggregation for at least 9 weeks at 4°C.

The invention also provides a method for suppressing an immune response in a mammal by the delivery of a therapeutic or effective amount of liposomal cyclosporin formulation. The formulations are useful for the treatment of autoimmune diseases and in the treatment of allograft patients. In another aspect of the present invention, a method is provided for reducing multiple drug resistance of a cancer cell by delivering a therapeutic or effective amount of a cyclosporin, preferably cyclosporine, liposomal formulation to a mammal. To effectuate the reduction in the multiple drug resistance of a cancer cell the cyclosporin is used in a treatment combination with an antineoplastic or anticancer agent. Although the cyclosporin is used in combination with

the antineoplastic or anticancer agent, it is not necessary for them to be delivered simultaneously. In yet another aspect of the invention, a method of inhibiting the growth of cancer cells, both drug resistant and drug sensitive, is provided by delivering a therapeutic or effective amount of free cyclosporin or liposomal cyclosporin to cancer cells, preferably in a mammal. Since dosage regimens for cyclosporins are well known to medical practitioners, the amount of the liposomal cyclosporin formulations which is effective or therapeutic for the treatment of the above mentioned diseases or conditions in mammals and particularly humans will be apparent to those skilled in the art.

This invention will be more fully understood by reference to the following examples, which are intended to be illustrative of the invention, and not limiting thereof.

Example 1

A series of liposome formulations containing cyclosporine were prepared. DSPC, cholesterol, DMPG and cyclosporine (21:1.5:3:1, 21:1:3:1, 24:1.5:3:1 and 24:1:3:1 respectively) were dissolved in a mixture of chloroform and methanol (1:1 by volume) and the solution formed was dried under nitrogen until a dried film was obtained. The lipid film was placed in a desiccator under vacuum for at least 8 hours to remove the residual organic solvent. The dried film was hydrated at 65°C for ten minutes in 10 mM sodium succinate in 9% sucrose pH = 6.75. Unilamellar vesicles were formed by sonicating the solution for 15 minutes at 65°C until a translucent solution was obtained. The solution was incubated at 65°C for 10 minutes. The solution was centrifuged at 3600 rpm for 10 minutes and the supernatant was collected. The concentration of lipids and cyclosporine was determined by HPLC. The size of the vesicles was determined by optical particle sizing. The results are listed in Table 1.

TABLE 1

ENCAPSULATIONS WITH VARYING MOLAR RATIOS OF CYCLOSPORINE

Mole Ratio (DSPC:Chol:DMPG: Cyclosporine)	% Cyclosporine Entrapped	Size by Mean Diameter (nm)
21:1.5:3:1	>90	38.0
21:1:3:1	>90	37.3
24:1.5:3:1	>90	46.7
24:1:3:1	>90	34.1

All samples of Table 1 show no visible signs of aggregation after nine weeks at 4°C.

Example 2

Multiple Drug Resistance (MDR) Testing in P388/Al)R Cells.

In order to test the effect of doxorubicin with cholesterol- containing formulations of liposomal cyclosporin A, the following experiment was done. Fifty microliters of the appropriate Doxorubicin (Dox) concentration was added to the wells of a flat bottom 96-well culture plate. In addition, 50 µl of liposomal formulations prepared as in Example 1, or free Cyclosporin A (CsA Crel), were added to the wells. All experiments were done in triplicate. The P388/ADR cells were grown 24 hrs in complete RPMI-1640 and then centrifuged at 1000 rpm for 10 min. The cell pellet was adjusted to 1×10^5 cells/ml in RPMI-1640 + 20% FCS + 2% FCS and 1% pen-strep, and the plates were incubated for 24 hrs at 37°C. (Positive control wells only contained cells and media, and negative control wells contained cells, media, and 20 µl of 1.5M Tris buffer). After 20 hours of incubation, the plates were pulsed with 0.5 µCi of ^{3}H-Thymidine per well and incubated for an additional 4 hours. Next, the cells were harvested using a Tomtec harvester and counted on a Betaplate scintillation counter. Linear regression analysis was used to determine the IC-50's of the various cyclosporin. A treatments and the doxorubicin IC-50 values were graphed as a function of CsA concentration.

The results of the tests are displayed in Table 2.

TABLE 2

Comparison of Cyclosporine-containing Formulations

Formulation	% Toxicity at 1800 ng/ml	Dox IC-50 at 1800 ng/ml of CsA
Free Drug	15%	446
(19:0:3:1)	30%	429
21:1.5:3:1	12%	308
21:1:3:1	10%	421
24:1.5:3:1	0%	477
24:1:3:1	0%	367

The cholesterol-containing liposomal formulations tested demonstrated a reversal of the MDR in P388/ADR cells. However, some of the formulations were more efficacious than others.

The formulations demonstrate low toxicity and good reversal of MDR.

Example 3

Immuno Suppressive Efficacy of 24:1:3:1 Formula

A DSPC:Chol:DMPG:CSA formulation (24:1:3:1) was prepared as described in Example 1. The following experiment was performed to compare the ability of various liposomal cyclosporin formulations, given *in vivo*, to suppress the splenic lymphocyte response to *in vitro* stimulation by concanavalin A (ConA) as compared to cyclosporine in complex L (CSA CreL, Sandimmune, SA). Mice were sacrificed and their spleens removed and placed in RPMI-1640 medium with 2% pen-strep (Gibco). A single cell suspension was made from each spleen by passing it through a 70 µm nylon mesh sieve (Falcon). The sieve was rinsed with RPMI+2% pen-strep to obtain a 10 ml volume of cell suspension. The splenic cell suspension was centrifuged at 1000 rpm for 8 min. and the supernatant was removed. The red blood cells were lysed with 1 ml lysing buffer (Cardinal Assoc. lot #09406 exp. Jan 1997) for 1 min., then centrifuged at 10000 rpm for 8 min. and the supernatant was removed. The cell pellet was resuspended in RPMI+5% FCS+l pen-strep, and cell viability determined by trypan blue staining. The cells were then adjusted to a cell concentration of about 5 x 10^6 cells/ml. Then the splenic lymphocytes were plated into the wells of a 96-well round bottom plate at 0.1 ml/well (5 x 10^5 cells/well).

Concanavalin A was diluted in PBS, aliquoted, and stored in a frozen state at -70° C prior to use. A 1 ml aliquot was thawed and diluted 1:4 in RPM+5 % FCS + 1 % pen-strep. The stock solution was diluted in RPMI+5 %FCS + 1 % pen-strep to obtain working solutions of 3 µCi ^{3}H-Thymidine (ICN Radiochemicals) per well on day 2. The cells were harvested onto glassfibre filtermats on day 3 using a Tomtec cell harvester. The filtermats were dried overnight, placed in sample bags with 10 ml Betascint cocktail, and the incorporated ^{3}H-Thymidine counted on a Betaplate scintillation counter (Wallac). The counts per minute (cpm) for triplicate wells were averaged, and the mouse treatment groups were averaged. The Δ cpm was calculated by subtracting the average cpm at ConA=O µg/ml from the average cpm at each ConA level. From these values, the percentage inhibition relative to the control was calculated by the following formula: % Inhibition = (Δ/cpm saline - Δ cpm experimental)/Δ cpm saline * 100.

A non-radioactive cell proliferation ELISA assay, the BrdU assay, was also performed on these samples. The plates were incubated as described above. On day 2, the wells were labeled with 20 µl/well of 100 µM BrdU labeling solution (5bromo-2'deoxyuridine; Boehringer-Mannheim) for 19 hours. The culture medium was removed by centrifuging the plates (300g, 10 min) and pipetting off 175 µl of medium. The plates were dried for 2.5 hr at 65°C. FixDent solution was added (200 µl/well) and incubated for

30 min at room temp (RT). The FixDent solution was removed and 100 µl/well of anti-BrdU-POD solution was added. The plates were incubated for 90 min at RT, then washed three times with washing buffer. The substrate solution was added (100 µl/well) for 7 min at RT. The substrate reaction was stopped by adding 25 µl/well of 1 M H_2SO_4. The absorbance was immediately measured at 450nm using a microplate reader (Titertek). The absorbance values for triplicate wells were averaged, and the mouse treatment groups were averaged. The Δ absorbance (A) was calculated by subtracting the average absorbance at ConA=O µg/ml from the average absorbance at each ConA level. From these values, the percentage inhibition relative to the control was calculated by the following formula: % Inhibition = (Δ saline - Δ A experimental)/ Δ A saline * 100

The following results were obtained. In the ^3H thymidine assay at 1.5 µg/ml ConA, 2.0 µg/ml ConA and 3.5 µg/ml the CSA-CreL showed approximately 5%, 12.5% and 2% inhibition, respectively. The liposomal formulation at the same ConA concentrations (24:1:3:1) showed 13%, 10% and 3% inhibition respectively.

In the BrdU assay CSA-CreL at 1.5 µg/ml ConA and 2.4 µg/ml ConA showed approximately 30%, 30%, and 70% inhibition respectively. The liposomal formulation (24:1:3:1) at the same ConA concentrations showed approximately 52 %, 52 % and 75 % inhibition respectively.

Example 4

Lipid films or spray dried powder containing various mole ratios of lipids and CSA as shown in Tables 3 and 4 were prepared. For the formulations shown in Table 3, DSPC was used. For the formulations shown in Table 4, HSPC was used. The formulations contained DSPC or HSPC, cholesterol, DMPG and cyclosporine A. The spray dried powder or lipid films were hydrated with an aqueous buffer containing 9% sucrose in 10 mM succinate at pH 6.5 and heated to 55°C and dispersed. This mixture was further hydrated in a water bath at 55°C with stirring for 30 minutes. Shear force was applied at temperatures above the transition temperature of the formulation with a modified Gaulin homogenizer (Gamble US Patent Number 4,753,788) at 4,000 - 12,000 psi to generate unilamellar liposomes having average median diameters (Microtract) as shown in Table 5. Samples were filtered at 50 - 65°C through a 0.22 µm filter composed of cellulose acetate.

TABLE 3

DSPC Based Liposomal Cyclosporin Formulations

DSPC Based Formulations (DSPC:cholesterol:DMPG:CSA)	Average Median Diameter (nm)
40:1:3:2	44.3
50:1:5:2	39.7
40:1:5:2	33.9
20:0.25:3:2	29.9
40:1:3:1.5	34.3
20:0.5:3:1.5	30.1
50:1:3:1	37.0
45:1:3:1	39.0
40:1:3:1	33.4
35:1:3:1	42.2
30:1:3:1	29.9
40:1:5:1	33.3
35:1:5:1	30.2
30:1:5:1	27.6
35:1:3:1	42.2
30:1:3:1	29.9
20:0.5:3:1	29.1
24:1:3:1	28.5
24:1:3:0.8	27.0
24:1:3:0.5	23.9
24:1:3:0.25	23.2
24:1:3:0.1	22.2

TABLE 4

HSPC Based Liposomal Cyclosporin Formulations

HSPC Based Formulations (HSPC:cholesterol:DMPG:CSA)	Average Median Diameter (nm)
20:0.5:3:1.5	29.0
35:1:5:1	39.8
20:0.5:3:1	30.1
40:1:3:1	62.1
36:1:3:1	33.1
34:1:3:1	37.1
32:1:3:1	35.1
30:1:3:1	38.1
28:1:3:1	31.4
26:1:3:1	34.1
24:1:3:1	34.2
24:1:3:0.8	30.1
24:1:3:0.67	31.0
24:1:3:0.5	29.7
24:1:3:0.25	25.7

Example 5

The protocol for ascertaining the percent of CSA remaining in the plasma/liposome fraction after incubation with rat whole blood is described below. Each liposomal cyclosporin formulation was analyzed by HPLC to determine the concentration of CSA. Rat (Fischer, male) whole blood was obtained via cardiac puncture and collected into tubes containing EDTA as an anticoagulant. To ensure that hemolysis and/or cell damage has not occurred from the phlebotomy, for each tube of blood collected a small aliquot was centrifuged at 10,000 rpm for 5 minutes in a Brinkmann Eppendorf 5415 centrifuge. The red blood cells formed a pellet at the bottom of the tube and the plasma remained in a separate layer above the red blood cells after treatment. The plasma fraction was examined for color. If the plasma color was straw yellow to light pink, the vial of whole blood was determined to be acceptable and used in the assay. If the plasma color was bright red it was determined that hemolysis had occurred and the affected tube of blood was be discarded. The whole blood tested was pooled to collect the appropriate volume of blood needed to complete the assay.

To each ml of whole rat blood, 0.6 mls of a cyclosporin containing liposome formulation was added to mimic an equivalent dose of 80 mg/kg in rats (1.7 - 2.0 mg/ml CSA). The exact amount of drug introduced (100% recovery) was calculated ([CSA] mg/ml *0.6 ml). The samples were immediately vortexed for approximately 10 seconds and incubated in a water bath at 37°C for 4 hours. After the incubation was complete each sample was vortex mixed again for about 10 seconds and centrifuged at 10,000 rpm for 5 minutes in a Brinkmann Eppendorf 5415 centrifuge. The top plasma layer was removed and transferred via a glass Pasteur pipette to a test tube and the exact volume of plasma was recorded. An aliquot of the plasma was then assayed for CSA by a HPLC. The CSA content was then calculated for the plasma/liposome fraction ([CSA] mg/ml in plasma * plasma volume(ml)/mg total CSA added* 100) and was expressed as a percent of CSA recovered. The results are displayed in Table 5. It should be noted that 10 - 12% of the plasma volume was occluded in the red blood cell pellet and therefore not recoverable. The data presented in Table 5 was not corrected for the occluded volume such that the maximal percent of CSA recovered was at most 88 - 90%.

TABLE 5

% CSA Detected in the Plasma Fraction After Four Hours

Liposomal Cyclosporin Formulation (PC:Chol:DMPG:CSA)	Type of PC	Sonicated or Homogenized	% CSA in Plasma/Liposome Fraction at 4 Hours
26:1:3:1	HSPC	homogenized	64
28:1:3:1	HSPC	homogenized	79
30:1:3:1	HSPC	homogenized	90
32:1:3:1	HSPC	homogenized	85
34:1:3:1	HSPC	homogenized	87
36:1:3:1	HSPC	homogenized	84
40:1:3:1	HSPC	sonicated	90
35:1:5:1	HSPC	sonicated	53
24:1:3:1	HSPC	sonicated	47
24:1:3:0.8	HSPC	sonicated	44
24:1:3:0.67	HSPC	sonicated	46
24:1:3:0.5	HSPC	sonicated	71
19:3:1	DSPC	sonicated	45
20:0.25:3:2	DSPC	sonicated	27
35:1:5:1	DSPC	sonicated	53
30:1:5:1	DSPC	sonicated	50
20:0.5:3:1	DSPC	sonicated	36
24:1:3:1	DSPC	sonicated	40
24:1:3:0.8	DSPC	sonicated	60
20:0.5:3:1.5	DSPC	sonicated	33
35:1:3:1	DSPC	sonicated	87
40:1:3:1	DSPC	sonicated	85
30:1:3:1	DSPC	sonicated	64

Example 6

Hemoglobin Release Test

It is desirable to develop a liposomal formulation of cyclosporin A that is stable and does not lose its active ingredient when introduced to whole blood. A hemoglobin release test was developed to screen candidate liposomal formulations

containing cyclosporine A. The assay involved quantitating the amount of hemoglobin (an indicator of red blood cell lysis or damage) present in a plasma fraction after incubation of rat whole blood with liposomal formulations of cyclosporin A at 37°C for 0, 1 or 4 hours followed by centrifugation at 10,000 rpm for 5 minutes.

The amount of hemoglobin released is related to the amount of loosely associated cyclosporin A in the liposomal formulations. A modified version of Sigma's Drabkin assay kit, which converts all oxidation species of hemoglobin to cyanomethemoglobin prior to quantitation, was used for the determination of total hemoglobin concentration in plasma.

The protocol for performing the hemoglobin release test is described below. Rat (Fischer, male) whole blood was obtained via cardiac puncture and collected into tubes containing EDTA as an anticoagulant. The ensure hemolysis and/or cell damage has not occurred from the phlebotomy, for each tube of blood collected a small aliquot was centrifuged at 10,000 rpm for 5 minutes in a Brinkmann Eppendorf 5415 centrifuge. The red blood cells formed a pellet at the bottom of the tube and the plasma remained in a separate layer above the red blood cells after subjection to this treatment. The plasma fraction was examined for color. If the plasma color was straw yellow to light pink the vial of whole blood was acceptable and used in the assay. If the plasma color was bright red hemolysis had occurred and the affected tube of blood was discarded. Whole blood was tested as previously described was pooled to collect the appropriate volume of blood needed to complete the entire assay.

To each ml of whole rat blood, 0.6 mls of a cyclosporin containing liposome formulation was added to mimic an equivalent dose of 80 mg/kg in rats (1.7 - 2.0 mg/ml CSA). A control sample was prepared with 1 ml of whole blood alone which was subjected to all the subsequent steps of the assay. The samples were immediately vortexed for 10 seconds and incubated in a water bath at 37°C for 4 hours. After the incubation was complete each sample was vortex mixed again for 10 seconds and centrifuged at 10,000 rpm for 5 minutes in a Brinkmann Eppendorf 5415 centrifuge or equivalent. The top plasma layer was removed and transferred via a glass Pasteur pipette to a test tube.

An aliquot of the plasma was assayed for hemoglobin concentration using a modified version of Sigma's Drabkin's test kit. Plasma levels of hemoglobin, determined after incubation of the CSA liposomes with rat whole blood for four hours and corrected for the control value, are presented in Table 6. For this test, lower concentrations (< 0.5 mg/ml) of hemoglobin released represents preferred formulation candidates. It should be noted that for formulations with a molar ratio of CSA lower than 1, less CSA is available in the formulation for the proposed cyclophilin interactions thus results for these samples may be favorably biased.

TABLE 6

Hemoglobin Release Test for Liposomal Cyclosporin Formulations

Liposomal Cyclosporin Formulation (PC:CholDMPG:CSA)	Type of PC	Sonicated or Homogenized	Hemoglobin Concentration in Plasma (mg/ml)
26:1:3:1	HSPC	homogenized	0.05
28:1:3:1	HSPC	homogenized	0.01
30:1:3:1	HSPC	homogenized	0.00
32:1:3:1	HSPC	homogenized	0.07
34:1:3:1	HSPC	homogenized	0.13
36:1:3:1	HSPC	homogenized	0.19
CSA in Cremphor EL	na	na	2.08
35:1:5:1	HSPC	sonicated	2.22
24:1:3:1	HSPC	sonicated	1.55
24:1:3:0.67	HSPC	sonicated	1.09
24:1:3:0.5	HSPC	sonicated	0.33
19:3:1	HSPC	sonicated	0.34
20:0.25:3:2	DSPC	sonicated	16.18
35:1:5:1	DSPC	sonicated	9.92
20:0.5:3:1	DSPC	sonicated	4.27
24:1:3:1	DSPC	sonicated	3.51
20:0.5:3:1.5	DSPC	sonicated	3.20
35:1:3:1	DSPC	sonicated	0.20
40:1:3:1	DSPC	sonicated	0.17
30:1:3:1	DSPC	sonicated	0.07

The data in Table 6 demonstrates that for optimal liposomal CSA formulations, which range from 28 - 40: 1 :3: 1 PC: cholesterol: DMPG:CSA, there is significant improvement in lower levels of hemoglobin released in plasma compared with CSA CreL. These improvements range from 10 fold improvement (35:1:3:1 DSPC: cholesterol: DMPG: CSA) to >200 fold improvement (30:1:3:1 HSPC: cholesterol: DMPG: CSA).

The preferred formulations, however, are shown by combining the criteria of Tables 3 & 4 which indicate that the following range of formulas would achieve the necessary whole blood stability and lower hemoglobin release: 28:1:3:1 to 40:1:3:1. The

preferred formulations being: 28:1:3:1, 30:1:3:1, 32:1:3:1, 34:1:3:1, 36:1:3:1, 5 and 40:1:3:1 with HSPC used as the preferred phosphatidylcholine.

Although the specification has been disclosed and illustrated with reference to particular applications, the principles involved are susceptible to numerous other applications which will be apparent to those skilled in the art. The invention is, therefore, to be limited only as indicated by the scope of the appended claims.

We claim:

1. Liposomes comprising a phosphatidylcholine, cholesterol, dimyristoylphosphatidylglycerol and cyclosporin wherein said liposomes are unilamellar having a size of less than 75 nanometers and are stable in whole mammal blood.
2. The liposomes as recited in claim 1 wherein the phosphatidylcholine is selected from the group consisting of distearoylphosphatidylcholine and hydrogenated soy phosphatidylcholine.
3. The liposomes as recited in claim 2 wherein the phosphatidylcholine is hydrogenated soy phosphatidylcholine.
4. The liposomes of claim 1 wherein the mole ratio of phosphatidylcholine to cholesterol to dimyristoylphosphatidylglycerol to cyclosporin ranges from about 28:1:3:1 to about 40:1:3:1.
5. The liposomes of claim 2 wherein the mole ratio of phosphatidylcholine to cholesterol to dimyristoylphosphatidylglycerol to cyclosporin ranges from about 28:1:3:1 to about 40:1:3:1.
6. The liposomes of claim 3 wherein the mole ratio of phosphatidylcholine to cholesterol to dimyristoylphosphatidylglycerol to cyclosporin ranges from about 28:1:3:1 to about 40:1:3:1.
7. The liposomes of claim 1 wherein the mole ratio of phosphatidylcholine to cholesterol to dimyristoylphosphatidylglycerol to cyclosporin is about 30:1:3:1.
8. The liposomes of claim 2 wherein the mole ratio of phosphatidylcholine to cholesterol to dimyristoylphosphatidylglycerol to cyclosporin is about 30:l: 3: 1
9. The liposomes of claim 3 wherein the mole ratio of phosphatidylcholine to cholesterol to dimyristoylphosphatidylglycerol to cyclosporin is about 30:1:3:1.
10. Liposomes having a size less than 100 nm comprising a phosphatidylcholine, cholesterol, dimyristoylphosphatidylglycerol and cyclosporin in a mole ratio from about 21:0.5:3:1 to about 21:1.5:3:1.
11. The liposomes of claim 10 wherein the phosphatidylcholine is distearoylphosphatidylcholine.
12. Liposomes having a size less than 100 nm comprising a phosphatidylcholine, cholesterol, dimyristoylphosphatidylglycerol and cyclosporin in a mole ratio of about 24:0.5:3:1 to about 24:1.5:3:1.
13. The liposomes of claim 12 wherein the phosphatidylcholine is distearoylphosphatidylcholine.

14. The liposomes of claim 12 wherein the mole ratio of phosphatidylcholine, cholesterol, dimyristoylphosphatidylglycerol and cyclosporin is about 24:1:3:1.

15. The liposomes of claim 13 wherein the mole ratio of distearoylphosphatidylcholine, cholesterol, dimyristoylphosphatidylglycerol and cyclosporin is about 24:1:3:1.

16. The liposomes of claim 10 wherein said liposomes are free of substantial aggregation for at least nine weeks at 4°C.

17. A method for preparing stable liposomal cyclosporin formulations, comprising:

a) (a) dissolving (i) a phosphatidylcholine, (ii) a cholesterol, (iii) a phosphatidylglycerol, and (iv) a cyclosporin in an organic solvent to form a solution wherein the molar ratios of (i):(ii):(iii):(iv) are as described above in claims 4, 8, 11 and 13;

(b) drying the organic solution thus formed to form a solid phase;

(c) hydrating the solid phase with an aqueous solution having a pH from about 4.5 to about 9.5 to form stable liposomal cyclosporin therapeutic formulations having a mean particle size less than 100 nm.

18. A method for treating a patient in need of immunosuppression, comprising administering to the patient an effective amount of a liposomal formulation according to any of claim 1-17.

19. A method for the treatment of drug resistant cancers, comprising administering to the patient in need of treatment thereof an effective amount of a liposomal formulation according to any of claims 1-17.

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 96/09053

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/127 A61K38/13

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 18104 (VESTAR, INC.) 29 October 1992	1-3
A	see claims 1-4,10-12	4-19

Y	JOURNAL OF PHARMACEUTICAL SCIENCES, vol. 79, no. 3, March 1990, WASHINGTON (US), pages 216-219, XP000101621 S. VENKATARAM ET AL.: "PHARMACOKINETICS OF TWO ALTERNATIVE DOSAGE FORMS FOR CYCLOSPORINE: LIPOSOMES AND INTRALIPID" see page 217, column 1, line 1 - line 7	1-3

P,A	EP,A,0 697 214 (VESTAR, INC.) 21 February 1996 see the whole document	1-19

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 October 1996	18. 10. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Benz, K

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 96/09053

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	DE,C,44 02 867 (DR. RENTSCHLER ARZNEIMITTEL GMBH & CO.) 14 June 1995 see claims 1-4	1-3

2

INTERNATIONAL SEARCH REPORT

In ational application No.
PCT/US96/09053

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 18, 19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/US 96/09053

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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